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Inventor(s)	:	Mark T. Fisher et al.
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Title	:	CHAPERONIN AND OSMOLYTE PROTEIN FOLDING AND RELATED SCREENING METHODS
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Commissioner for Patents
P.O. Box 1450
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DECLARATION OF DR. MARK FISHER UNDER 37 C.F.R. § 1.132

Commissioner for Patents
Washington, D.C. 20231

I hereby declare as follows:

1. My name is Dr. Mark Fisher, MD. I have a Ph.D. in Biochemistry from the University of Illinois, Urbana IL. Ph.D., 1987.

2. I am a co-inventor in the above-captioned patent application.

3. I have been employed by the University of Kansas Medical Center since 1992. Since 1997, I have been employed as Associate Professor. Department of Biochemistry and MolecularBiology University of Kansas Medical Center ("KUMC"), the assignee of the above-captioned invention.

4. Protein folding is defined as the process whereby the hydrophobic parts of a flexible polymer become buried inside the interior as the flexible polymer collapses to a more

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organized and defined three dimensional structure. Protein folding is therefore a complex reaction that can chose multiple routes at the same time. Thus, correct protein folding involves a balance between these multiple folding reactions (generically called pathways) that lead to correct and functional proteins (enzymes, structural proteins etc.) vs. those routes that lead to off-pathway incorrectly folded proteins. In many cases, where proteins often misfold, it is because the off-pathway routes are faster than the routes that lead to correctly folded proteins.

5. The precise order of this chaperonin/osmolyte protein folding process of the present invention is crucial to insure successful screening for optimal conditions. The folding protein is initially captured by the oligomeric chaperonin to form an arrested chaperonin-protein substrate complex. Applicant has repeatedly shown that this arrested form can hold to protein in a metastable but eventually foldable state for a long period of time (~2 hr at 37°C; Fisher, 1992). Once this complex is formed, the test osmolyte solution(s) can then be added to the arrested chaperonin-protein substrate complex and released into the test osmolyte solution where the ability of the osmolyte to influence successful protein folding can be evaluated. The order of addition is crucial because there are numerous instances where osmolyte addition to a folding substrate alone prior to forming the arrested chaperonin-protein complex results in large scale protein misfolding (Voziyan et al., 2000; Voziyan and Fisher, 2000, attached to November 24, 2003 IDS). For example, Figures 4A and 6 in the latter reference show the glutamine synthetase misfolds (no activity, increase in aggregation species) when TMAO alone (without the chaperonin) is added to refolding glutamine synthetase monomers. Thus, to specifically practice the Applicant's invention, it is necessary to first form the complex between protein (e.g. ΔV468 GS, malate dehydrogenase, rhodanese or citrate synthase) and chaperonin (e.g. GroEL) and then add the test osmolyte (e.g. urea or TMAO or glycerol etc (see list in Table 1)) and ATP to determine if added osmolyte will allow the protein to acquire its correct folded structure once it is released from the chaperonin.

6. In the present invention, low concentrations of one of the osmolytes tested, urea (~1 M), enables one to control the speed of the misfolded pathways, which in turn prevents these misfolding pathways from taking over and forcing all or most of the folding protein to go down the wrong path. Functionally, the high affinity chaperonin is able to bind partially folded proteins before the proteins get into trouble. Once the protein is released from the chaperonin, it still can choose between multiple paths for folding (misfolding vs. folding). When urea present, misfolding reactions down the wrong path (which is usually the faster collapse pathway) slows down when the protein is released from the chaperonin. If the protein does not fold to its proper correct (forms a protected oily interior) within a certain time frame, the protein will rebind to the chaperonin and it can repeat this process over and over again. Thus, the present invention involves a situation where slowing the misfolding protein down will enable more protein to fold to its native state. In short, urea actually helps the chaperonin folding platform capture and release the folding protein by preventing the off-pathway folding.

7. Evidence of this is shown in **FIG. 7** of the patent specification. In **FIG. 7**, folding is helped by urea because urea prevents the folding intermediate from rapidly collapsing to a misfolded form, allowing the chaperonin to bind it and efficiently fold it even when the folding protein is present in excess (more the 1 molecule folding protein per 1 chaperonin - the stoichiometry). Furthermore, the experiments highlighted in Figure 7 showed that the inclusion of urea with the chaperonin enhanced the regained activity of Rhodanese above the chaperonin alone. With urea alone, at 37°C the folding of rhodanese is extremely poor (See Weber et al., 1998, IDS Figure 3B). This figure shows that the chaperonin urea combination exhibits the synergy that is unique to the process outlined in the patent specifications.

8. I have read and am familiar with the subject matter of the Grovotis Article (1997) and the Altamirano Articles (1997, 1999) cited in the above-captioned application.

9. In the Gorovits Article, urea was used only used to denature the protein itself, not to promote the folding of the polypeptide.

10. The Gorovits Article merely investigated whether differing denaturing conditions (urea vs. thermal) would change the chaperonin requirements (GroEL, GroES ATP vs. GroEL GroES ADP). The reference simply created a different denatured protein to bind to the chaperonin by using either heat or urea to denature the protein. The folding of the protein DHFR was initiated in the presence of GroEL with residual urea present. However, the inclusion of urea did not in any way ***promote*** the folding of DHFR or activity regain to its native conformation or near native activity levels as required by the claimed invention.

11. GroEL (without nucleotide) actually suppresses the spontaneous successful folding of dihydrofolate reductase ("DHFR"). See Viitanen et al., "*Complex Interactions between the Chaperonin 60 Molecular Chaperone and Dihydrofolate Reductase*," Biochemistry pp 9716-9723 (1991) (cited in the Gorovits Article). GroEL only has the ability to release DHFR after ATP is added to the arrested DHFR-chaperonin complex. The released DHFR does not gain any advantage of folding yield with the chaperonin present with urea present because it does not require the chaperonin to fold unlike the particular protein substrate that was used (ΔV468 GS) in Applicant's patent application.

12. In sum, in the Gorovits Article, urea is not used as a folding aid for the protein in question at all. Furthermore, the protein in question folds very nicely by itself without requiring the chaperonin. The urea that is present during the folding reaction is only there because the unfolded protein in concentrated urea was diluted into the solution containing the chaperonin. The Gorovits Article does not teach or suggest the use urea to enhance the stiochiometry between the folding protein and the chaperonin. In order to teach this particular method, Gorovits and Horowitz would have had to show that the inclusion of urea with the chaperonin offered an advantage for folding (yields for example) compared to the chaperonin alone or urea alone. This was not demonstrated nor can it be inferred from the data. However, the present invention used rhodanese as a substrate and shows that the inclusion of urea with the chaperonin enhances the protein folding yields and allows one to use a lower concentration of the chaperonin to achieve

high yields (See Figure 7- patent specification). We also used a large number of other osmolytes (Table I) to test the broad array of test conditions this chaperonin/osmolyte system offers.

13. The Altamirano Articles use a mini-chaperonin system. The minichaperone system is inferior to and will not function with even some of the most commonly used polypeptides that chaperonins fold. In this regard, the Wang et al., (1998) and Weber et al., (1998) articles included in the Information Disclosure Statement show that the Altamirano minichaperone system fails to fold various stringent chaperonin requiring protein substrates. In cases where successful folding with the minichaperone is observed, Altamirano and his coworkers refolded one stringent class III chaperonin dependent protein, but only under conditions where rhodanese can fold by itself (so-called "permissive" 25° C folding conditions). See Wang (1998) Figure 3; Smith & Fisher (1995) Figure 1. Thus, the minichaperone fails to fold this protein to any degree under very controlled conditions (Wang et al., 1998, Figure 3 A and B in this figure - sht345 is the minichaperone fragment apical domain). Curiously, casein a milk protein that is not even classified as a chaperone does better than the minichaperone fragment.

14. In particular, Wang et al., (1998) and Weber et al., (1998) showed that the minichaperone system could not function to fold malate dehydrogenase or the maltose binding protein. The minichaperone fails to fold its test substrate Rhodanese at 37°C (See Weber et al., (1998) Figure 3 . In contrast, as illustrated in Example 8, the present chaperonin/osmolyte invention is capable of working with other substrates (such as malate dehydrogenase) See also (Tieman 2001). Further, subsequent work by the inventors (Voziyan and Fisher) indicates that the present invention involving chaperonin/osmolyte systems shows that it works with citrate synthase, another one of the test substrates that the minichaperones will fail to fold. Furthermore, the present invention is able to fold various chaperonin substrates at much higher protein concentrations at physiological temperatures (37°C) than any of the in vitro methods available (Table 2) without excessive misfolding and aggregation (Fisher 1993 listed in patent specification). The primary difference between the claimed invention, which uses the oligomeric version of the chaperone, and that of the minichaperone fragment is that the latter cannot capture and hold folding polypeptides or stabilize a metastable protein state for any length of time.

15. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

Date: April 23, 2004 By: Mark T Fisher